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Structural diversity of secretory products in the glandular parts of the oviduct of two plethodontid salamanders (Amphibia, Urodela)

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A b s t r a c t. Semithin and in particular ultrathin sections of the glandular subdivisions in the oviduct of the terrestrial egg laying, direct developing plethodontid salamanders *Bolitoglossa pesruba* and *Oedipina uniformis* revealed remarkable structural differences of secretory products between the two species and in *B. pesrubra* between the different subdivisions of the duct. In the latter species structure of the secretory granules confirmed the previously described histological differentiation of the glandular portion of the oviduct in four subdivisions. In the first subdivision most secretory granules were moderately electron-dense having a distinct osmiophilic core, in the second these cores are absent, in the third granules revealed a complex inner structure and in the fourth they are more or less homogeneous and electron dense. In *O. uniformis*, however, secretory granules were differently stained in semithin sections with toluidine blue, but showed a homogeneous moderately electron-dense appearance along the entire oviduct. As oviductal secretions form the generally multi-layered glycoproteinaceous egg jelly enveloping the ovum when passing down the oviduct, our results suggest that in terrestrial breeders considerable differences exist in these secretions even at the structural level and, therefore, very likely in number and nature of egg jelly layers.

Key words: oviduct, secretory granules, ultrastructure, plethodontids, terrestrial eggs

Introduction

The oviduct in urodelan amphibians provides the ovulated egg with a glycoproteinaceous jelly that consists of a varying number of layers (=egg capsules). The number of oviductal glandular subdivisions largely corresponds with the number of these jelly layers that, among other aspects, aid in gamete recognition, binding of spermatozoa, mechanical support, etc. (for further readings see G r e v e n 2002, 2003). Depending on the species considered, the spawning site and the systematic relationships, number, consistency and even hydration of certain egg jelly layers show considerable variation (S a l t h e 1963). Such variations may be reflected at least partly in the structure of the oviduct, and, particularly, in the nature of its secretory products. This has been documented within Urodela for viviparous species and a few species that lay aquatic eggs (for further readings see G r e v e n 1998, 2002, 2003).

Studies that document oviductal structure and secretions in urodeles with otherwise derived reproductive modes are largely missing (see W a k e & D i c k i e 1998). In a previous paper we described the oviduct of five species of plethodontid salamanders that deposit their eggs in terrestrial sites, where the offspring develops directly. External appearance of the oviduct and simple staining for acidic and neutral glycoconjugates enabled us to distinguish several subdivisions in the glandular part of the oviduct (=*pars*

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convoluta excluding the uterine portion that was aglandular). Gland cells in the different subdivisions often showed heterogeneous AB- and PAS-reactivity even in the same cell and gland cells contained both AB- and PAS-positive granules (E h m c k e et al. 2002/2003).

Among the different techniques used for the study of amphibian oviducts, transmission electron microscopy (TEM) may be a useful tool to clarify subdivision by analysing the structural diversity of the glandular secretions (B o i s s e a u 1973, 1979, 1980; G r e v e n 1980). However, such investigations are rare and available only for three salamandrid species that lay either aquatic eggs or are viviparous. Direct developers such as the Bolitoglossinii with their terrestrial eggs have not been investigated in this respect (summarized by G r e v e n 1998, 2002, 2003).

We had sufficient material to study the main subdivisions of the oviduct by TEM from two nationally protected, terrestrial egg-laying plethodontid species, *Bolitoglossa pesrubra* and the related *Oedipina uniformis*, that were collected in the field, As the general structure of the oviduct is similar in all urodeles, we focus herein on the ultrastructural aspect of the secretions of its glandular part that show a surprising heterogeneity.

Material and Methods

One female of *Bolitoglossa pesrubra* (= *B. subpalmata*) (snout vent length 5.7 cm; largest ovarian eggs 2 mm in diameter) and one female of *Oedipina uniformis* (snout vent length 6.4 cm, largest ovarian eggs 3 mm in diameter) both collected in Costa Rica at the beginning of May 1999 (capture permission Nr. 117.98-OFA, export permission Nr. DGVS-199-98) were processed some days later for electron microscopy in the laboratory (Unidad de Microscopía electrónica, Universidad de Costa Rica, San José, Costa Rica).

Small portions of oviductal subdivisions identified by gross morphology (see E h m c k e et al. 2002/2003) were excised and fixed in 2.5 % glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.2. Later in the laboratory the tissue was postfixed in 2% osmiumtetroxide in the same buffer, dehydrated in ethanol and embedded in Spurr's medium (S p u r r 1969). For orientation 1 µm-semithin sections were made and stained with toluidine blue/borax. Ultrathin sections were made with a diamond knife, stained with uranyl acetate (W a t s o n 1958) and lead citrate (R e y n o l d s 1963), and viewed in a Zeiss EM 900. Due to their consistency, some parts of the oviduct of *O. uniformis* could not be sectioned. In these cases we cut sections ca. 0.25 µm thick and viewed them at 100 kV.

Results

The urodele oviduct is a long convoluted tube that begins cranially with the ostium tubae and opens caudally with its "uterine" part in the cloaca. From outside to inside it consists of the thin peritoneal epithelium, a more or less pronounced muscle layer, vascularized connective tissue and a mono-layered epithelium. As described previously, the oviduct of the two species examined herein can be divided into six longitudinally pleated subregions, the aglandular *pars recta* and the *pars convoluta* that exhibits four glandular subdivisions in *Bolitoglossa pesrubra* and five in *Oedipina uniformis*, and the aglandular uterine portion. All oviductal subdivisions are lined by a simple epithelium that is columnar in the glandular subdivisions (E h m c k e et al. 2002/2003).

The epithela of the *pars recta* and the "uterine" part consists of ciliated and non-ciliated cells and are largely similar in both species; they will not further treated herein. Both cell

types, however, occur also along the glandular oviduct; here at the crests of the longitudinal folds (see Figs 6, 7, 21). The glandular part was well developed in the two females and gland cells were crowded with secretory products that pushed the large euchromatic nuclei at the base or at the margin of cells (Figs 3, 5, 7, 13, 17, 19, 21). Cell organelles including dictyosomes and rough endoplasmic reticulum (rer) were localized basally and in the spaces between secretory granules. We describe the most abundant and "typical", very likely mature secretory granules of the main subdivisions and mention some others that differ from them.

Bolitoglossa pesrubra

Semithin sections of the oviduct reveal four subdivisions in the glandular *pars convoluta* (Figs 1, 3, 5, 7).

Pars convoluta 1: Gland cells are crowded with numerous secretory vesicles that were stained with toluidine blue in different intensities ranging from very pale to dark blue (Fig. 1). The round granules touch one another but appear not to be distorted and do not coalesce (Fig. 2). Only small areas of cytoplasm are visible that contain cell organelles, mainly dilated cisterns of rer and large dictyosomes (not pictured). Diameter of secretory granules ranges from 3 to 4 µm; they are characterized by a heterogeneous matrix that contains at least one, occasionally compound, core of varying electron density that is visible already in semithin sections (Figs 1, 2). Cores may change their appearance in posterior direction becoming more loosened and the matrix appears more homogeneous (Fig. 9).

Pars convoluta 2: Semithin sections again show differently stained granules (Fig. 3). Secretory granules have a diameter of 4 to 6 µm and do not possess a core; their matrix is more homogeneous (Fig. 4). Cell organelles (e.g. mitochondria, rer and dictysomes) are confined to small spaces between the granules.

Pars convoluta 3: In the main part toluidine blue-staining is more homogeneous (Fig. 5). At the fine structural level granules (diameter 4 to 5 µm) again lack a core, but the matrix shows a complex ultrastructure (Fig. 6). In addition, granules with an obviously disordered inner structure occur (Fig. 10). Cytoplasmic areas reveal predominantly stacks of dictyosomes and some profiles of rer.

Pars convoluta 4: In this subivision granules are highly distinct and heavily stained in semithin sections (Fig. 7). At the ultrastructural level most granules have a very electron dense matrix that appears loosened in some cases (Fig. 8) or have a more electron-light area (Fig. 11). Most posteriorly granules are homogeneous and touch one another so that they become distorted (Fig. 12). Granule diameter ranges from 2 to 4 µm. Between the granules remarkably dilated cisterns of rer are present (Figs 8, 22).

Oedipina uniformis

Judging from semithin sections, organisation of secretory granules and stainability with toluidine blue justifies subdividing of the *pars convoluta* (Figs 13, 15, 17, 19). At the ultrastructural level, however, subdivision is less clear (Figs 14, 16, 20).

In semithin sections secretory granules are stained with different intensities; at the base of the glandular epithelium, secretory granules often are unstained and have an irregular outline (Fig. 19). Although ultrathin sections could not be obtained from these regions, 0.25 µm sections showed an aspect similar to the adjacent stainable granules.

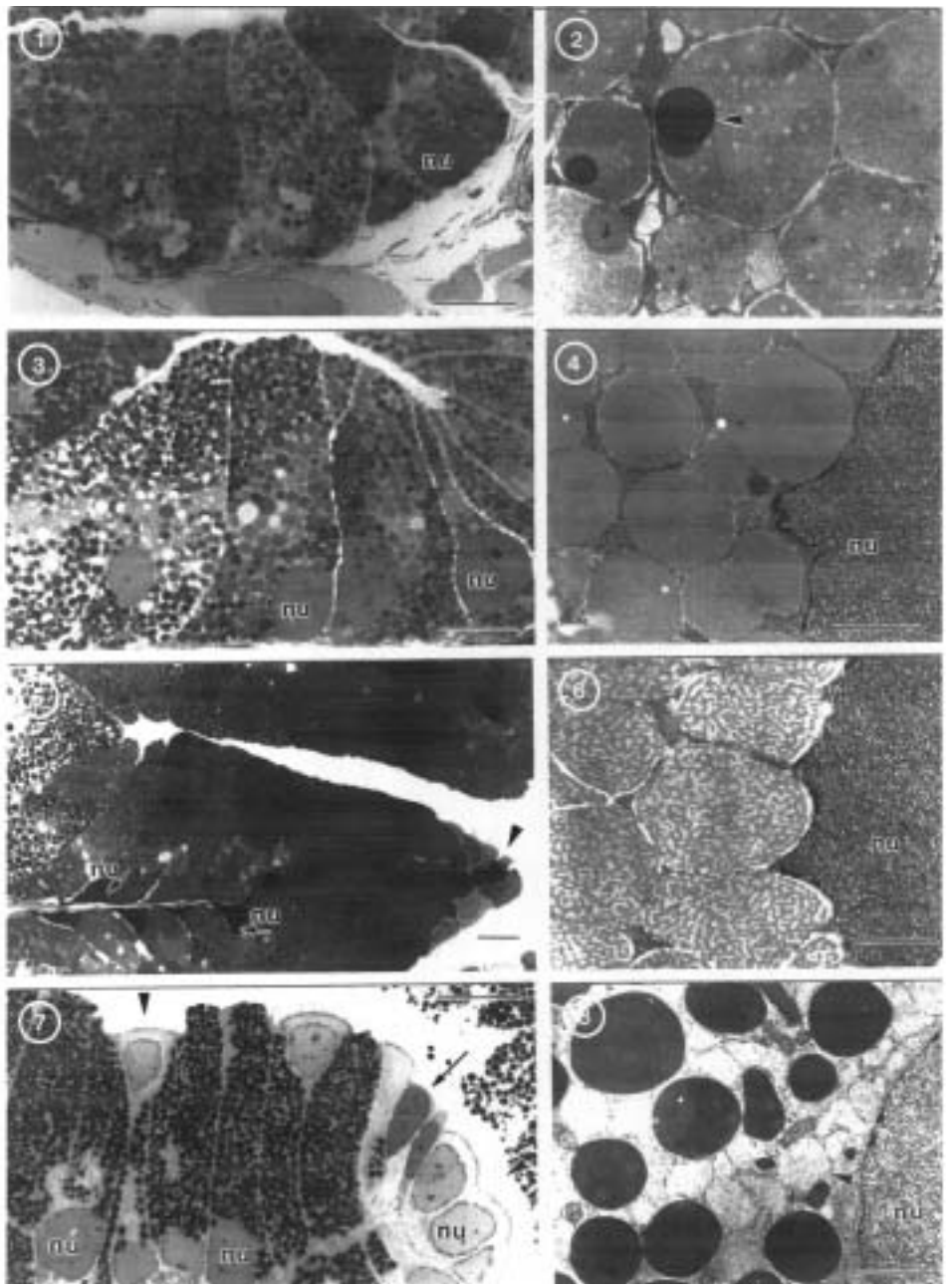

Fig. 1.–8. The glandular parts of the *pars convoluta* (*p.c.*) in the oviduct of *Bolitoglossa pesrubra*. 1, 3, 5, 7 semithin sections stained with toluidinblue borax. Scale bar 20 µm. 2,4,6,8 ultrathin sections of secretory granules. Scale bar 2 µm. **1,2** *p.c.* 1; note cores in the secretory granules (1) that are partly compound and of different electron density (arrowhead) (2). **3,4** *p.c.* 2; differently stained granules and euchromatic basal nuclei (3); note the homogeneous matrix of secretory granules (4). **5,6** *p.c.* 3; secretory granules are homogeneously stained in semithin section (5), but have a complex inner structure in ultrathin sections (6). Ciliated cell (arrowhead). **7,8** *p.c.* 4; note distinct granules in the gland cells, large euchromatic nuclei and ciliated (arrowhead) and non-ciliated cells (arrow) (7), between the electron dense secretory granules dilated cisterns of rer (arrowhead) are seen (8); nu nucleus

The ultrastructural analysis showed that gland cells are filled with densely packed homogeneous granules of scant electron density (Figs 14, 16, 18, 20, 22). They touch one another, especially in the first subdivision, where they are partly distorted (Fig. 14) and seem to coalesce in the second (Fig. 16). In the small spaces between granules dilated cisterns of rer are present.

Discussion

In a previous study we distinguished six subdivisions in the oviduct of *Bolitoglossa pesrubra* and seven in *Oedipina uniformis* on the basis of gross morphology, histology and simple carbohydrate histochemisty (AB, PAS-staining): the aglandular most anterior *pars recta*, and

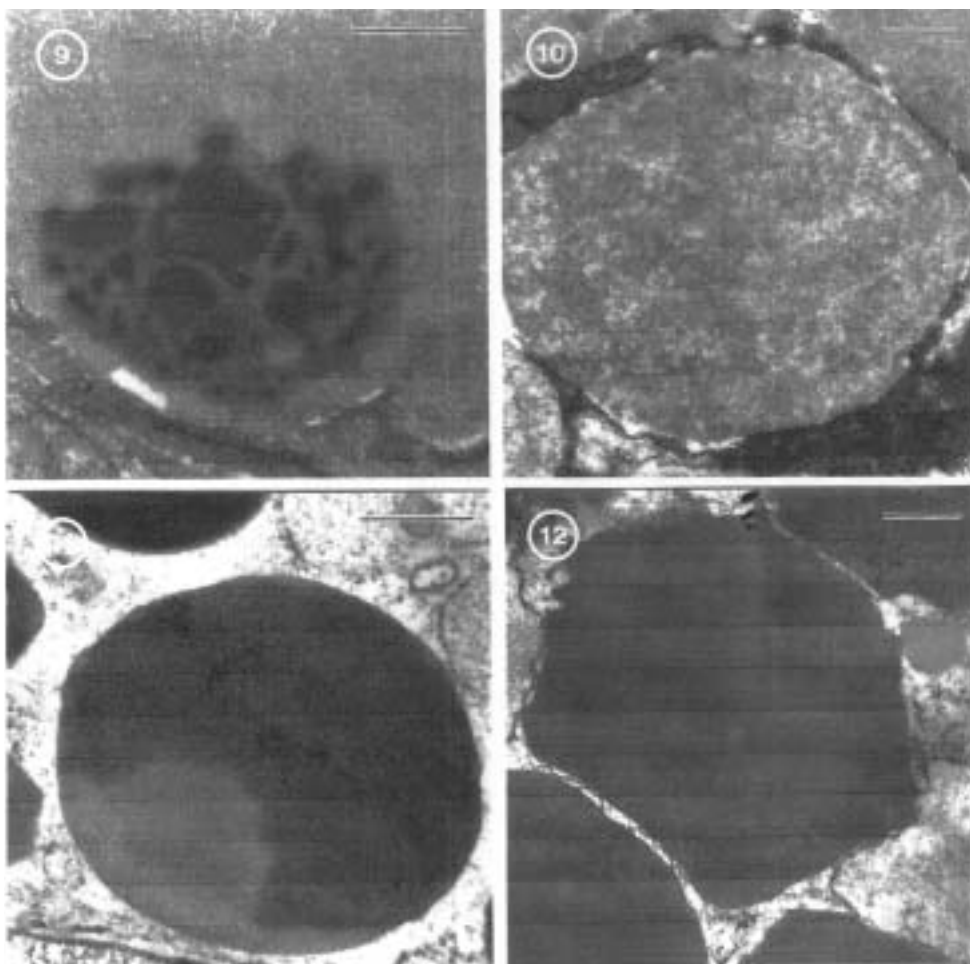

Fig. 9-12. Ultrastructural aspect of secretory granules that differ from "typical" ones in the *pars convoluta* (*p.c.*) of *Bolitoglossa pesruba*. Scale bar 0.5 µm. **9** *p.c.* 1, posteriorly; note the loosend core. **10** *p.c.* 3; note the disordered content. **11** *p.c.* 4; note the areas of different electron density. **12** *p.c.* 4, more posteriorly; note granules that touch one another.

four or five glandular subdivisions plus the aglandular uterine part in the *p. convoluta* (Ehmcke et al. 2002/2003).

A largely aglandular short *p. recta* appears to be present in all urodelan oviducts investigated as yet. An aglandular "uterine" portion does occur in viviparous species, where it is enlarged and houses the developing offspring, but also in some oviparous species including all plethodontids hitherto investigated. The non-ciliated cells in both the *p. recta* and the "uterine" portion produce moderate amounts of glycoproteins of unknown function. Ciliated cells (and smaller non-ciliated) are distributed along the entire oviduct. Motion of cilia may help to distribute secretions and to move the egg (Greven 1998, 2002, 2003).

The glandular parts of the oviduct of all plethodontids hitherto investigated are lined by a simple columnar epithelium; as yet only one species was found to have tubular glands in the first subdivision (for review see Greven 2002, 2003; Ehmcke et al. 2002/2003). The two females examined in the present study had an inconspicuous fat body and eggs obviously near maturation. In the *B. pesrubra* specimen investigated the largest follicles in the ovary measured 2 mm in diameter. This value corresponds with that of mature eggs of the same species given by Vial (1968). Smaller follicle diameters (ca. 1.5 mm) in a second specimen corresponded with a thinner, less convoluted oviduct (Ehmcke et al. 2002/2003). In *O. uniformis* the largest follicle measured even 0.5 mm in diameter and the large ovaries were seen through the transparent abdomen. Such large follicles indicate a forthcoming spawning that needs fully developed oviductal glands. Also if oviductal and ovarian activity should not be synchronized exactly in the plethodontids examined, a quiescent stage of the oviduct far beyond vitellogenesis appears improbable, because urodeles in general resume synthesis of glycoproteins earlier (see also Greven 2003). In the aseasonal breeder *B. pesrubra* Vial (1968) and Wake & Dickie (1998) described fully developed oviducts all over the year. Oviductal glands of the both females investigated by us were extremely crowded with secretory granules, which does not, however, exclude further synthesis of new material as suggested by the large Golgi-Apparatus (not pictured) and the often dilated cisterns of rer.

Gland cells are often distinguished by the physicochemical properties of their secretions, and oviductal gland cells have been classified commonly as mucous with secretions containing proteins and sugars. Usually, presecretory mucigen droplets are formed by the dictyosomes, gradually increase in size and may change their appearance. Thus, some of the smaller and/or differently stained granules and granules with a content more disordered than the "typical" ones may represent different stages of maturation. However, discussion about the development and maturation of oviductal secretion is beyond the topic of this study (for details see Boisseau 1973, 1979, 1980). Whether fully mature or not, secretory granules in *B. pesrubra* are remarkably heterogeneous and elaborated. This heterogeneity allows subdivision of the glandular part of the oviduct at the level of secretory granules and confirms largely previous attempts to subdivide the oviduct of this species (Ehmcke et al. 2002/2003). In addition, some sections of a developing egg in this species revealed a multi-layered egg capsule (Greven 2003).

Secretory granules in the oviduct of *O. uniformis* are of a surprising structural homogeneity along the entire length of the glandular *p. convoluta*. Thus, subdividing on the basis of their structure is nearly impossible. Gross morphology, histology and the carbohydrate staining mentioned above show, however, clear regionalization. Similarly, secretory granules along the entire glandular oviduct of the highly specialized *Siren lacertina*

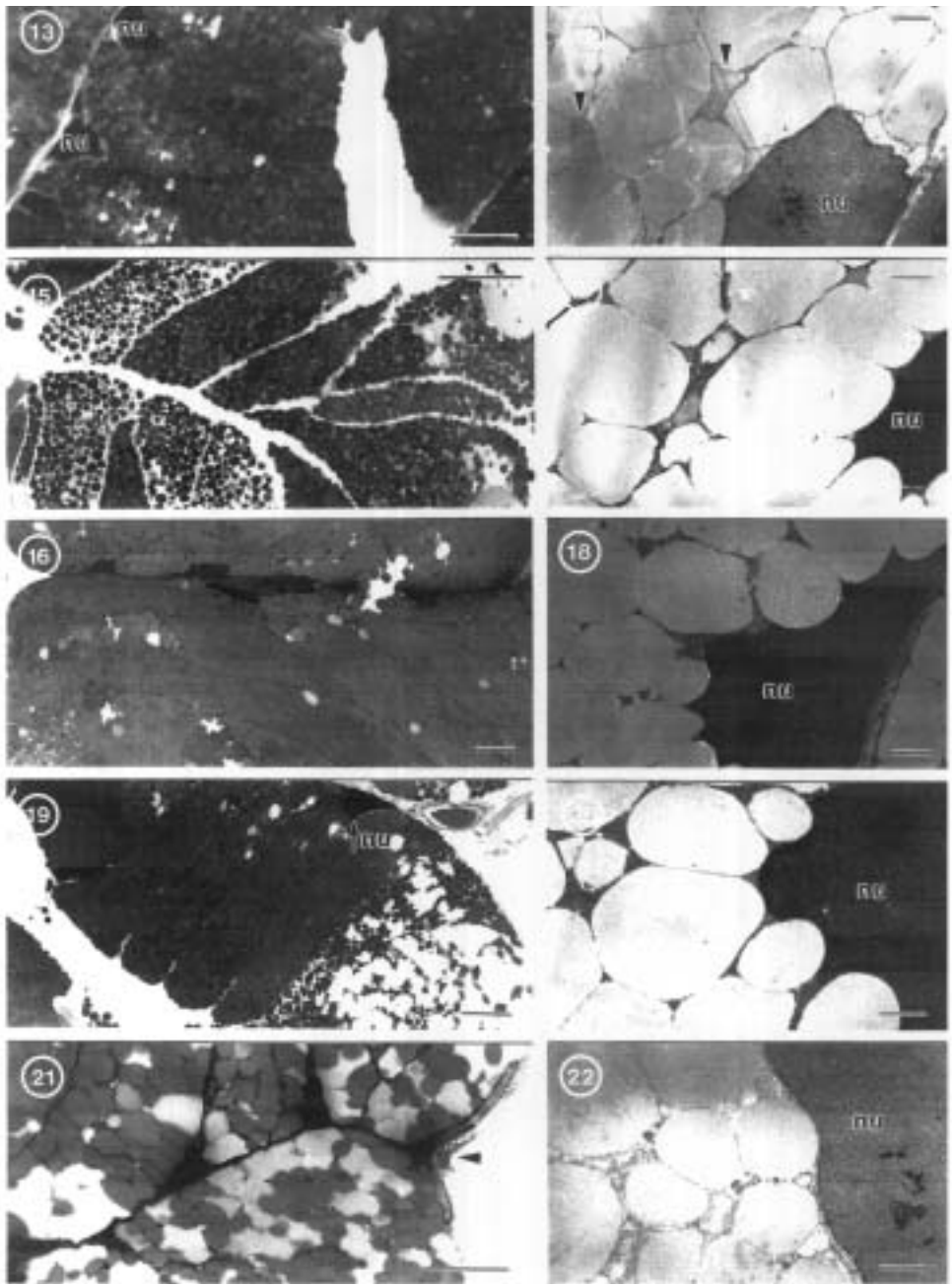

Fig. 13-22. The glandular parts of the *pars convoluta* (*p.c.*) in the oviduct of *Oedipina uniformis*. 13, 15, 17, 19, 21 semithin sections stained with toluidinblue borax. Scale bar 20 µm. 14, 16, 18, 20, 22 ultrathin sections of secretory granules. Scale bar 2 µm. **13,14** *p.c.*1; note secretory granules not clearly distinct, but differently stained with toluidinblue (13); secretory granules touch one another and are partly distorted (14); dilated rer (arrowheads). **15,16** *p.c.* 2; note distinct granules (15) that are largely electron lucent and coalesce partly (16). **17,18** *p.c.* 3; more homogeneously stained secretions (17) of low electron density (18). **19,20** *p.c.* 4, darker stained granules; note unstained granules (asterisk) (19) and their electron-lucent aspect (20); ciliated cell (arrowhead). **21,22** *p.c.* 5; heterogeneously stained granules (21) that are very electron-lucent (22); nu nucleus

have a homogeneous and electron-lucent matrix (S e v e r et al. 1996). Also in that species gross anatomy and very likely histology of the oviduct showed subdivisions (S e v e r et al. 1996) and egg jelly analysis revealed more than one distinguishable layer or capsule (S a l t h e 1963). This surely holds also for the eggs of the rare and burrowing *O. uniforms*.

The glycoprotein-secreting gland cells of the urodelan oviduct have been classified as mucous and seromucous in the oviparous *Pleurodeles waltlii* on the basis of histochemical reactions and their ultrastructural aspect. Mucous oviductal cells, e.g. in the cranial part of the oviduct, were characterized by alcianophilia; electron-lucent, coalescing secretory granules; abundant dictyosomes; rough endoplsamic reticulum; and seromucous cells, e.g. in the middle part of the oviduct, by a strong positive PAS-reaction; electron-dense non coalescing uniformly sized granules digested largely by pronase; and dictyosomes and a well-organized rough endoplasmic reticulum (B o i s s e a u 1973, 1979, 1980). Electron dense cores in secretory granules appear to have a greater portion of proteins. This is suggested by cytochemical studies on similarly complex granules in the oviduct of the related *B. dofleini* (E h m c k e et al., unpublished). With the exception of the fourth subdivision in the oviduct of *B. pesruba*, which might be considered as seromucous, the other oviductal gland cells herein described, in particular those of *O. uniformis*, escape from such a classification at the moment.

In brief, the ultrastructure of secretory products in the fully developed oviducts of the two plethodontid species examined herein show a remarkable diversity. In *B. pesrubra*, diversity along the oviduct confirmed subdivisions recently described on the basis of its external appearance and histology. In *O. uniformis*, structural homogeneity of the secretory products along the oviduct does not reflect clearly the subdivisions seen with these techniques. Our findings give evidence that in plethodonids, considerable taxon-specific structural and, thus, very likely chemical differences (not investigated as yet) occur in oviductal secretions regardless of the fact that all glands produce glycoconjugates in general, and that both species examined herein lay terrestrial eggs.

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